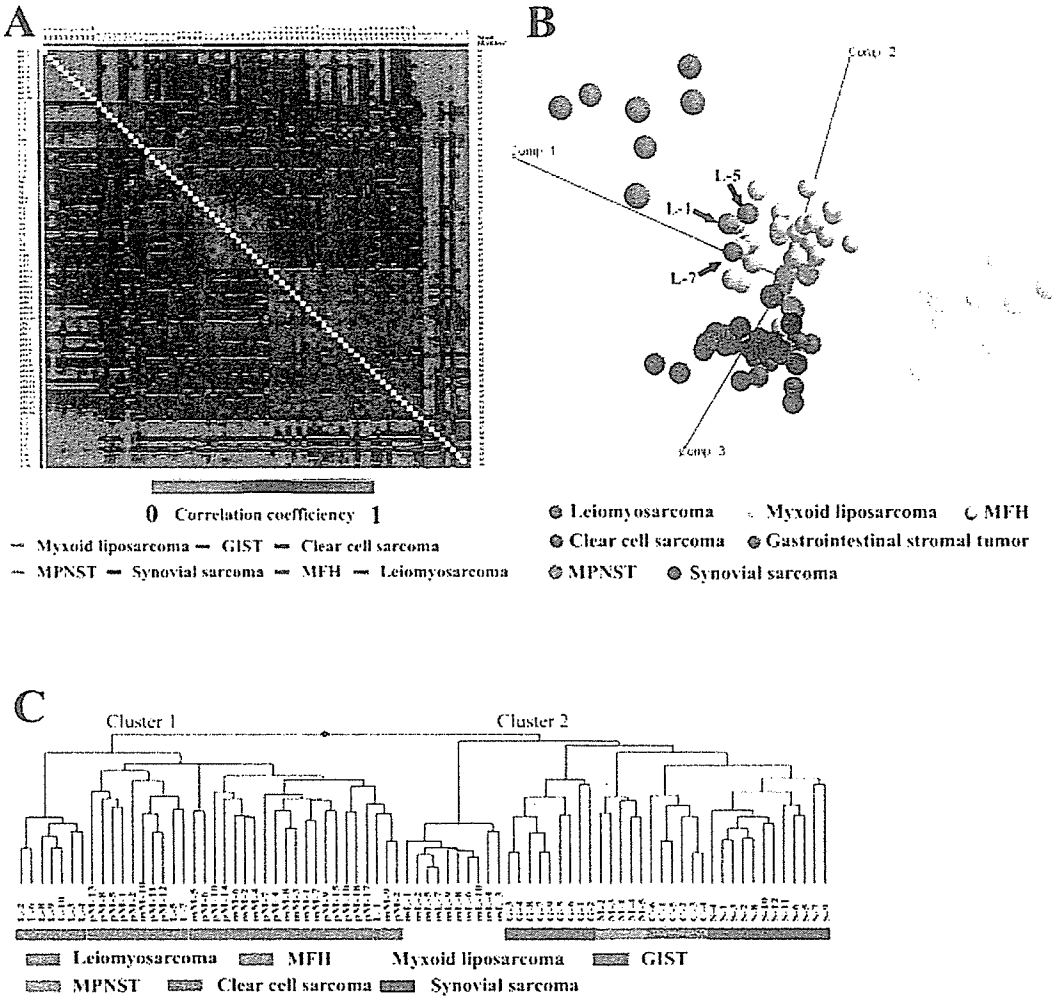

Figure 2. Classification of soft-tissue sarcoma by the selected 67 spots. (A) Correlation matrix using the protein expression profiles. (B) Principal component analysis of the 80 soft-tissue tumors on the basis of the expression profiles of the 67 proteins. Arrows indicated three pleomorphic leiomyosarcomas (L-1, L-5, and L-7). (C) Hierarchical classification on the basis of a distance tree constructed from the 67 proteins. The name of the 67 proteins used for the classification and their expression level were described in Supplemental Figure 6.

selection is demonstrated in Supplementary Fig. 10 and the results of protein identification are summarized in Supplementary Table 2). Hierarchical clustering using these five proteins divided 38 tumor samples into two groups (Fig. 3B). Nine of 11 samples in Cluster 1 showed histological grades I/II, and 25 of 27 samples in Cluster 2 were categorized in grade III. Although tumors with grades I/II and those with grade III did not show significantly different patient survivals in our study (p = 0.0857, Fig. 5A), the patients in Cluster 1 and those in Cluster 2 showed distinct survival rates (p = 0.0296, Fig. 5B). Hierarchical clustering subdivided Cluster 2 into two groups: Clusters 2a and 2b (Fig. 3B). The difference in patient survival between Clusters 1 and 2a was only suggestive (p = 0.0811). In contrast, patients in Cluster 2b had a significantly worse survival relative to those in Clusters 1 and 2a (p = 0.0028 and 0.0271, respectively; Fig. 5C).

4 Discussion

Identification of molecular signatures corresponding to histological subtypes is an essential step toward understanding of the molecular basis of tumor development. We generated protein expression profiles of 80 soft-tissue tumors from seven histological backgrounds and identified protein clusters unique to the histology. We identified a set of 67 proteins that showed distinctive expression patterns in the subclasses of soft-tissue sarcomas. Hierarchical clustering demonstrated that leiomyosarcomas and MFHs shared similar expression pattern of these 67 proteins (Fig. 2C and Supplementary Fig. 6). DNA microarray study also showed that these two tumors had similar gene expression profile [4]. The recent studies with comparative genomic hybridization (CGH) revealed that leiomyosarcoma and MFH had similar CGH imbalance profiles [19]. These observations suggested

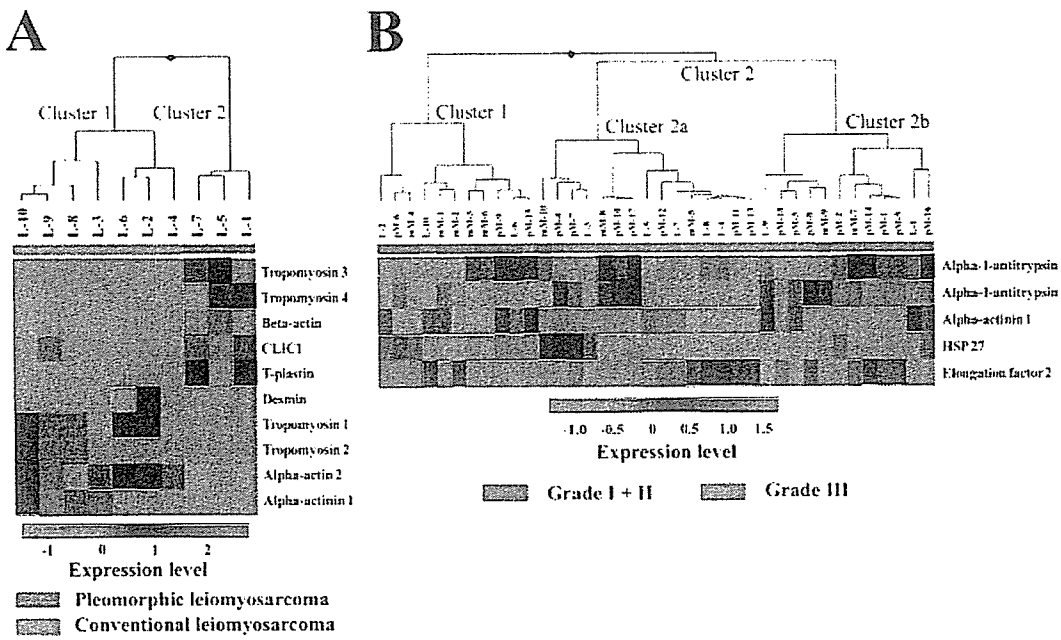

Figure 3. (A) Classification of leiomyosarcomas from expression of ten selected proteins. (B) Histological grading of leiomyosarcoma and MFH from expression of five proteins.

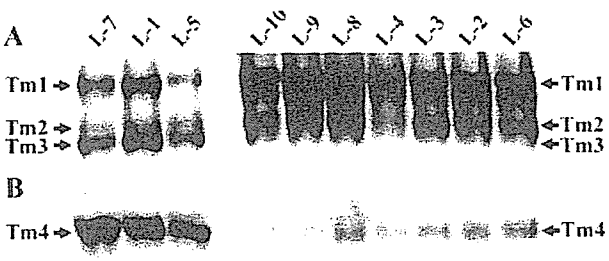

Figure 4. Western blotting for tropomyosin isoforms with specific antibodies. Specific antibodies against tropomyosin 1, 2, and 3 (A) and against tropomyosin 4 (B) were reacted with the leiomyosarcoma samples blotted on the membrane. As the antibody for tropomyosin 1, 2, and 3 did not distinguish these three isoforms, identification was achieved by the electrophoretic migration of the bands according to a previous report [32].

that these tumor types might correspond to different differentiation states of a single tumor. In our study, pleomorphic leiomyosarcoma was distinct from conventional leiomyosarcoma and close to MFH in terms of protein expression (Fig. 2B and C and Supplementary Fig. 6), probably reflecting different biological behavior and proliferating activities [20]. We noted that GIST, MPSNTs, clear cell sarcomas, and synovial sarcomas had similar protein expression profile (Fig. 2C and Supplementary Fig. 6). The similarity of these four tumor types is consistent with the biological association and similar gene expression of these tumor types. For example, clear cell sarcoma is known as melanoma of the soft parts, which was proposed to arise from a progenitor neural crest cell with the potential for melanocytic differentiation and melanin synthesis [21]. Gene expression study showed that synovial sarcoma and MPNST shared differential expression of several genes characteristic of neural crest-derived cells [5]. GIST may be derived not from the muscle cells in the intestinal tract but from neuronal cells with pacemaker activity in the bowel wall [22]. Gene expression profiling suggested the close association of GIST with synovial sarcoma [4]. These observations lead to the idea that the status of genome, transcriptome, and proteome may be reflected by the histological appearance of tumors and the identification of most critical genes or proteins for the classification will provide the diagnostic molecular markers for soft-tissue sarcoma.

We demonstrated that the expression levels of five protein spots including HSP27 were associated with histological grading and patient survival in leiomyosarcoma and MFH. HSP27 expression is associated with a favorable prognosis in MFH, correlating with overall survival and metastasis-free survival [23]. The prognostic value of HSP27 has also been suggested in many other types of cancer, including ovarian carcinoma [24], brain tumor [25], and gastric cancer [26]. Expression of the other proteins has also been correlated with the malignant characteristics of tumors. Up-regulated production of alpha-1-antitrypsin mRNA has been reported in pancreatic cancer [27] and liver cancer [28]. Modulation of alpha-actinin levels affected cell motility and conferred tumorigenicity on 3T3 cells [29]. In our study, multiple isoforms of alpha-actinin 1 were identified as informative proteins for histological classification, leiomyosarcoma subclassification, and histological grading (Supplementary Table 2). These isoforms were distinguished as different

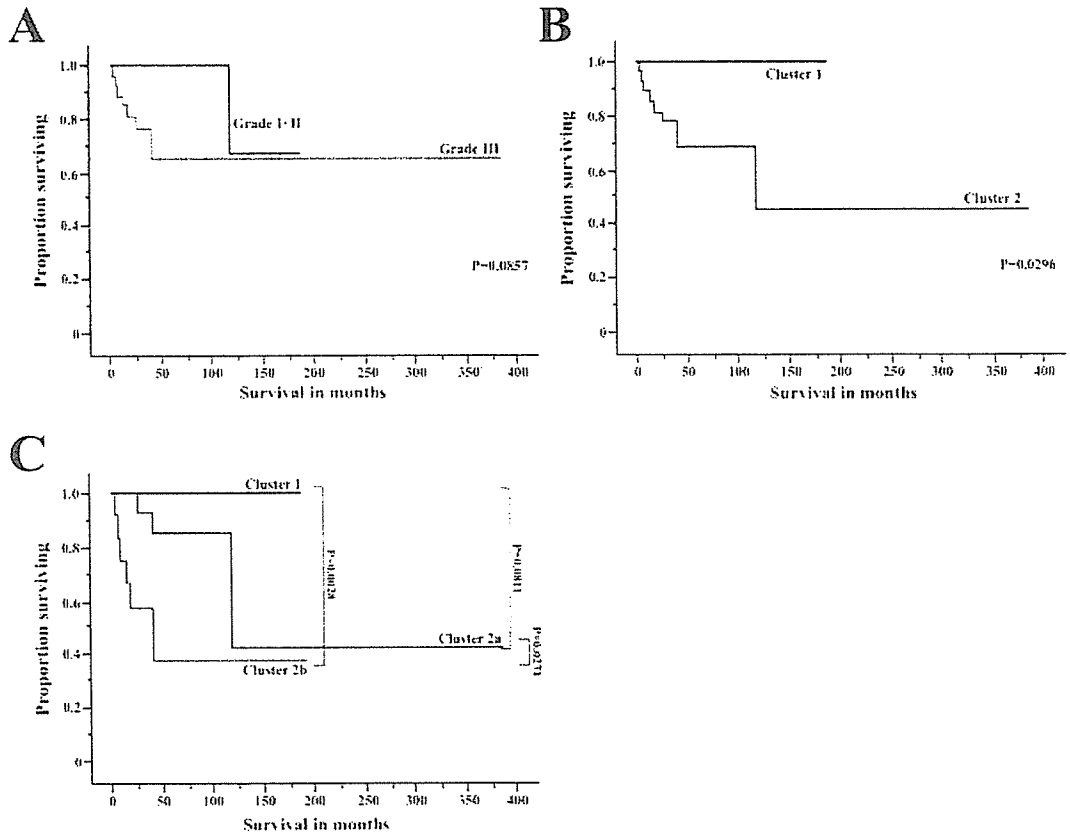

Figure 5. Kaplan–Meier survival curves for the groups with histological grading I/II and III (A) and for the groups of Clusters 1 and 2 in Fig. 3B. (B) Kaplan–Meier survival curves were also plotted for the groups of Clusters 1, 2a and 2b (Fig. 3B). *p* values were obtained by the log-rank test.

spots on the 2-D image and probably arise from PTMs. These isoforms may have different functional properties that contribute to the histological appearance and biological behavior of the tumors. Proteins associated with survival can be considered as a candidate for prognostic tumor marker, by which we can delineate a high-risk group that may benefit from adjuvant therapy and exclude low-risk patients in whom additional therapies are unlikely to exhibit clinical benefit.

We did not identify several known correlations between certain genes and tissue types, including between KIT and GIST [4], between SSX and synovial sarcoma [30], and between melanoma antigens and clear cell sarcoma [31]. This discrepancy is probably a result of the limited sensitivity of 2-D DIGE. As 2-D DIGE detects proteins nonspecifically according to amount, the expression levels of these oncoproteins may be below the LOD. Extensive efforts are being directed toward uncovering a greater fraction of the proteome by improving the sensitivity of current proteomic technologies, and such efforts will further benefit our understanding of the biology of soft-tissue sarcomas.

Genome-wide protein expression profiling will lead to the identification of important proteins and to novel molecular classifications. Our experiments revealed the proteins associated with histological classification and grading. The diagnostic and prognostic performance of the identified proteins will be validated in larger numbers of patients. As these proteins could also be involved in the development of soft-tissue sarcomas, study of their expression might contribute to novel therapeutic strategies.

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The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication. The corresponding author declared that there is no conflict of interest.

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A pilot study to assess the safety and efficacy of carbon dioxide insufflation during colorectal endoscopic submucosal dissection with the patient under conscious sedation

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Background: Endoscopic submucosal dissection (ESD) is accepted as one of the treatments for en bloc resection of large superficial colorectal lesions. This procedure is performed by using air insufflation, is time consuming, and is associated with severe abdominal discomfort. The safety and efficacy of carbon dioxide (CO_2) insufflation during colonoscopy already has been assessed in some trials.

Objective: To assess the safety and efficacy of CO_2 insufflation instead of air insufflation during colorectal ESD with the patient under conscious sedation.

Design: A case-control series with a historical control.

Patients: A total of 35 consecutive patients were enrolled in this study. Another 35 consecutive patients who previously received colorectal ESDs by using air insufflation were included as a historical control.

Interventions: Arterial partial pressure of CO_2 (pCO_2) was measured before and after each procedure with the total dose of midazolam used as an index of abdominal discomfort.

Main Outcome Measurements and Results: The mean (standard deviation [SD]) operation time was 90 ± 57 minutes in the CO_2 group and 100 ± 80 minutes in the control group (not significant). In the CO_2 group, the mean (SD) dose of midazolam was significantly lower than that of the control group; 5.6 ± 4.9 mg and 9.7 ± 5.9 mg, respectively ($P = .005$). Blood analysis revealed a slight pCO_2 elevation in the CO_2 group; however, only 2 patients complained of mild abdominal discomfort.

Limitations: Abdominal discomfort and pCO_2 were not evaluated in the control group.

Conclusions: This study strongly suggests that CO_2 insufflation is safe and effective during lengthy colonic endoscopic procedures, eg, ESD, with the patient under conscious sedation.

It has been claimed that carbon dioxide (CO_2) insufflation reduces patient pain and abdominal discomfort during and after colonoscopy. The safety and efficacy of CO_2 insufflation during colonoscopy was already assessed in some earlier trials.[1-7] Air insufflation is still the standard method, however, because of a lack of suitable equipment and continued technical improvement in colonoscopy.

Endoscopic submucosal dissection (ESD) with conventional air insufflation is accepted as one of the treatments for en bloc resection of selected large superficial colorectal lesions,[8-14] but this procedure is a lengthy one, where a considerable amount of air enters the colonic lumen and causes severe intraoperative and postoperative abdominal discomfort. CO_2 insufflation, therefore, may be preferable for longer colonoscopic procedures, eg, ESD.

In laparoscopic surgery, CO_2 insufflation is safely used with the patient under general anesthesia,[15-18] but there are no reports about its use in longer endoscopic procedures, such as colorectal ESD with the patient under conscious sedation. The aim of this study was to assess the safety and efficacy of CO_2 insufflation during colorectal ESD with the patient under conscious sedation.

PATIENTS AND METHODS

Patients

Between November 2004 and May 2005, a total of 35 consecutive patients were enrolled in this study at the National Cancer Center Hospital (NCCH) in Tokyo. All ESD

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procedures were performed by one or the other of 2 highly experienced colonoscopists (Y.S., T.U.).

Colorectal ESDs with air insufflation performed in 35 other consecutive patients by the same 2 colonoscopists between September 2003 and November 2004 were compared as a historical control group. Patients with severe chronic occlusive pulmonary disease and known CO_2 retention were excluded from the study.

Endoscopic operating system

Procedures were performed with Olympus video colonoscopes (Olympus Optical Co, Ltd, Tokyo, Japan). CO_2 was administered by using a commercially available CO_2 regulator (Gas Regulator, Crown, Model FR-IIS-P; Yutaka Engineering, Tokyo, Japan), which was connected to a CO_2 bottle.

ESD procedures

The margins of the lesion were delineated before ESD by using 0.4% indigo carmine dye spraying (Fig. 1A). After the submucosal injection of glyceol (Chugai Pharmaceutical Co, Ltd, Tokyo, Japan),[19] a circumferential incision in the mucosa was made with a needle knife[9-12] (Fig. 1B). A sodium hyaluronate solution[9-14] was then injected into the submucosal layer to lift the lesion, and the thickened submucosal layer was cut with a needle knife or an insulation-tipped knife[8] (Fig. 1C to E). The operation time was recorded for all patients.

Arterial partial pressure of CO_2 measurements

Arterial partial pressure of CO_2 (pCO_2) was measured before and after the procedure in all patients enrolled in the CO_2 group. Arterial pCO_2 was not measured in the historical control group.

Sedation

In all cases, midazolam (2 mg, intravenous) was used before insertion of the colonoscope. In addition, 2 mg was given repeatedly when indicated, based on the individual endoscopist's judgment.

Evaluation of abdominal discomfort

A questionnaire about the presence of abdominal discomfort (none, mild, moderate, and severe) was completed immediately after the ESD procedure for all patients in the CO_2 group.

Statistical analysis

All the variables in this study are described as mean and standard deviation (SD). To compare the baseline characteristics between the 2 groups, we used the t test for continuous variables and the χ^2 test for dichotomous variables. All statistical analyses were performed by using SAS version 8.0 (SAS Institute Inc, Cary, NC). The P values were 2 sided; $P < .05$ was used to determine statistical significance.

Ethics

The ethics committee of the NCCH approved the study protocol. Informed consent was obtained from all patients in the CO_2 group before entering the study.

RESULTS

There were no differences in clinicopathologic characteristics between the groups (Table 1). The mean (SD) size of the resected specimens was 32 ± 15 mm in the CO_2 group and 30 ± 14 mm in the control group, with no statistical difference (NS). En bloc resection was achieved in 30 of 35 cases (86%) in the CO_2 group and 31 of 35 cases (89%) in the control group.

Histopathologic evaluation revealed 5 low-grade dysplasias, 24 high-grade dysplasias, and 6 submucosal (sm) carcinomas in the CO_2 group. In comparison, there were 1 low-grade dysplasia, 22 high-grade dysplasias, and 12 sm carcinomas found in the control group.

Curative resection, defined as an en bloc resection of an intramucosal or sm superficial cancer (<1000 μm), without lymph vascular invasion or poorly differentiated component, was achieved in 89% (31/35) of the patients in the CO_2 group and 80% (28/35) of the patients in the control group, respectively. All margins were free of neoplasia.

Operation time and arterial pCO_2 in the CO_2 group

The mean (SD) operation time was 90 ± 57 minutes in the CO_2 group and 100 ± 80 minutes in the control group (NS). Mean (SD) arterial pCO_2 was slightly elevated (4.5 ± 5.4 mm Hg) in the CO_2 group; however, no major adverse effects were identified. As shown in Figure 2, arterial pCO_2 had a slight tendency to increase during longer procedures, but no statistical difference was found.

Dosage of midazolam

The mean (SD) dosage of midazolam in the CO_2 group was significantly lower than that of the control group, 5.6 ± 4.9 mg and 9.7 ± 5.9 mg, respectively ($P = .005$). Even after being adjusted for the difference in operation time, the dosage of midazolam in the CO_2 group was lower than that of the control group ($P = .05$).

Abdominal discomfort and complications

Two of 35 patients in the CO_2 group (5.7%) complained of nausea and mild abdominal discomfort after the procedure, but these patients had no symptoms after 1 hour. There were no perforations in the CO_2 group, but 3 perforations and 1 case of subcutaneous emphysema were observed in the control group; however, all 4 of these cases were treated endoscopically, without surgery.

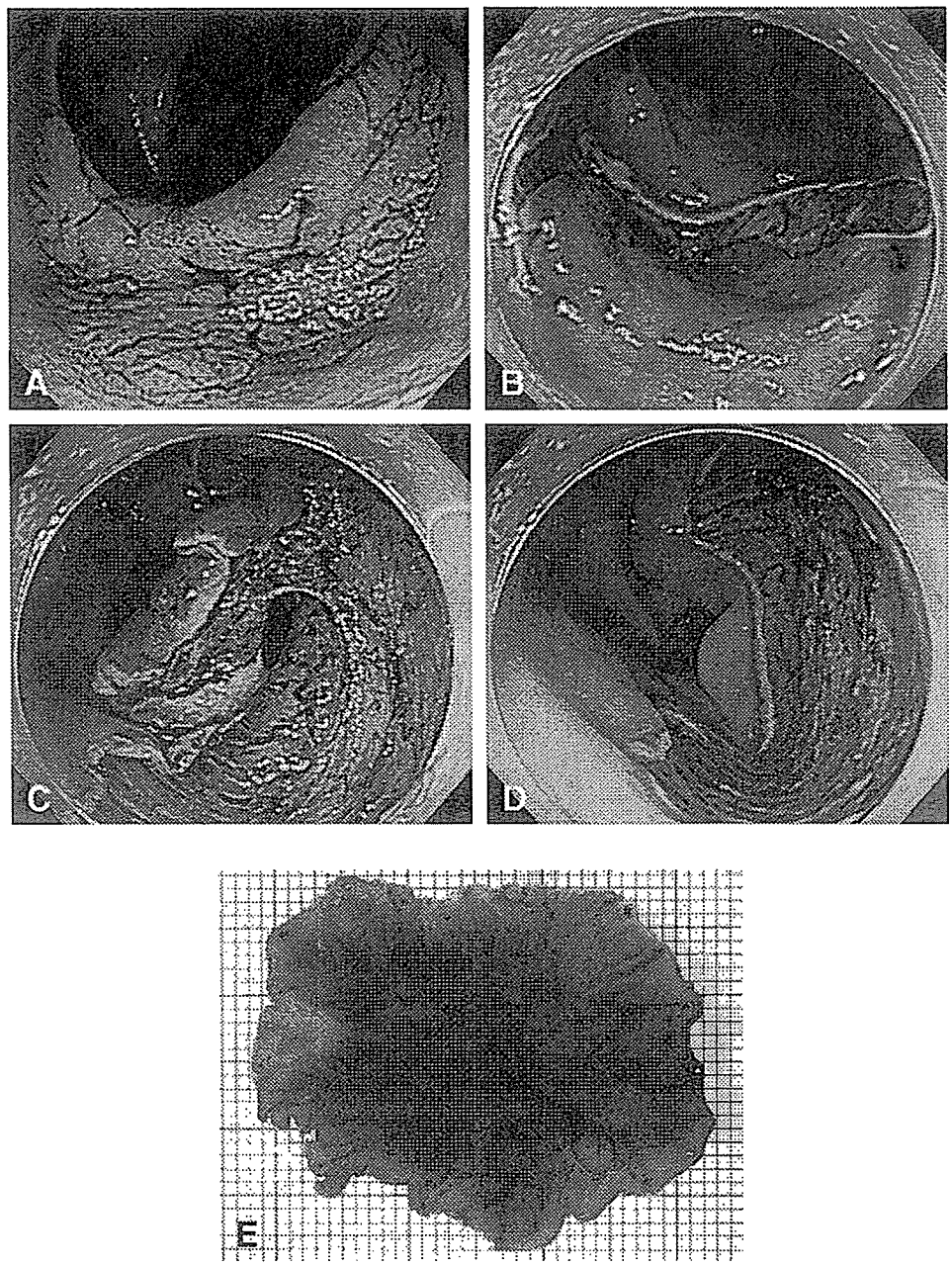

Figure 1. The ESD procedure. **A,** The margins of the lesion were delineated before ESD by using 0.4% indigo carmine dye spraying. **B,** After the injection of glyceol, a circumferential incision in the mucosa was made with a needle knife. **C,** Sodium hyaluronate solution was then injected into the submucosal layer to lift the lesion, and the thickened submucosal layer was cut with an insulation-tipped knife. **D,** The area of the colon after the en bloc resection. **E,** The resected specimen, 40 mm in diameter, revealed a high-grade dysplasia, whereas, by histologic evaluation, the resected margin was free of tumor.

DISCUSSION

Based on the results of this pilot study, CO_2 insufflation proved to be safe and effective during lengthy colonic endoscopic procedures (eg, ESD) with the patient under conscious sedation. Patient discomfort was considerably lower in the CO_2 group, probably because of a more rapid absorption of CO_2 than was caused by conventional air insufflation, as evidenced by the lower total dosage of midazolam.

In the field of conventional colonoscopy, the safety and efficacy of CO_2 insufflation has already been assessed in earlier studies, including randomized controlled trials.[1-7] Furthermore, in laparoscopic surgery, CO_2 insufflation is safely used for longer operations with the patient under general anesthesia,[15-18] but there are no reports about its

TABLE 1. Clinicopathologic characteristics between groups

	CO_2 group	Control group	*P* value
No. cases	35	35	
Tumor size (mean [SD]), mm	32 ± 15	30 ± 14	NS
Operation time, min	90 ± 57	100 ± 80	NS
Arterial pCO2 elevation, mm Hg	4.5 ± 5.4		
Dose of midazolam, mg	5.6 ± 4.9	9.7 ± 5.9	.005
Histopathology			
Low-grade dysplasia	5	1	
High-grade dysplasia	24	22	
sm1 (<1000 μm)	3	7	
sm2 (≥1000 μm)	3	5	
En bloc resection rate	30/35 (86%)	31/35 (89%)	NS
Free margin rate	25/35 (71%)	30/35 (86%)	.04
Curative resection rate	31/35 (89%)	28/35 (80%)	NS
No. complications			
Perforation	0	3	NS
Emphysema	0	1	NS

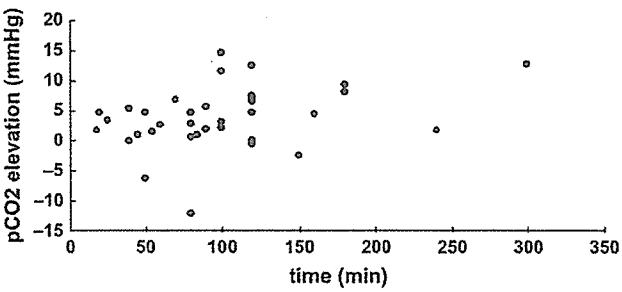

Figure 2. Arterial pCO_2 elevation (mm Hg). There was a slight tendency for arterial pCO_2 to increase when the operation time was longer, but there were no statistical differences.

extended use for more than 1 or 2 hours in colonoscopic procedures with the patient under conscious sedation.

In this study, CO_2 insufflation did not lead to a clinically significant increase in pCO_2 levels. This was most likely because of the conscious sedation, even in patients undergoing a lengthy colonoscopic procedure. It is well known that sedatives may cause hypoxemia and hypoventilation. Similarly, Bretthauer et al[7] reported, in a trial that involved conventional colonoscopy examinations, that sedated patients in both CO_2- and air-insufflation groups had a slight increase in end-tidal CO_2. Sedation and not CO_2 insufflation, therefore, is the primary cause of CO_2 retention during colonoscopy.

A limitation of this pilot study was that abdominal discomfort and pCO_2 were not evaluated in the control group. According to the dose of midazolam used, however, patient discomfort was considerably lower in the CO_2 insufflation group than with usual air insufflation. In addition, abdominal radiographs were occasionally performed on a limited basis after the procedure, and our experience indicated that there was often substantially less air in the CO_2 group (Fig. 3).

As for complications, there were no perforations in the CO_2 group, but 3 perforations and 1 case of subcutaneous emphysema were observed in the control group. One reason for this lower complication rate might be related to the progressing skill of the 2 colonoscopists, because the CO_2 group procedures were performed up to 6 months after the control group procedures.

Usefulness of ESD

ESD for early gastric cancer is widely accepted as a useful endoscopic treatment[20-29]; however, ESD for colorectal cancer is not widely accepted, because of its technical difficulty and the greater risk of perforation.[8-14]

Laterally spreading tumors (LSTs) >20 mm are usually treated by piecemeal resection (EPMR), and local recurrence is frequently observed after EPMR.[30,31] In this study, most of the resected tumors were >20 mm, but the en bloc resection rate with the ESD technique was 87%,

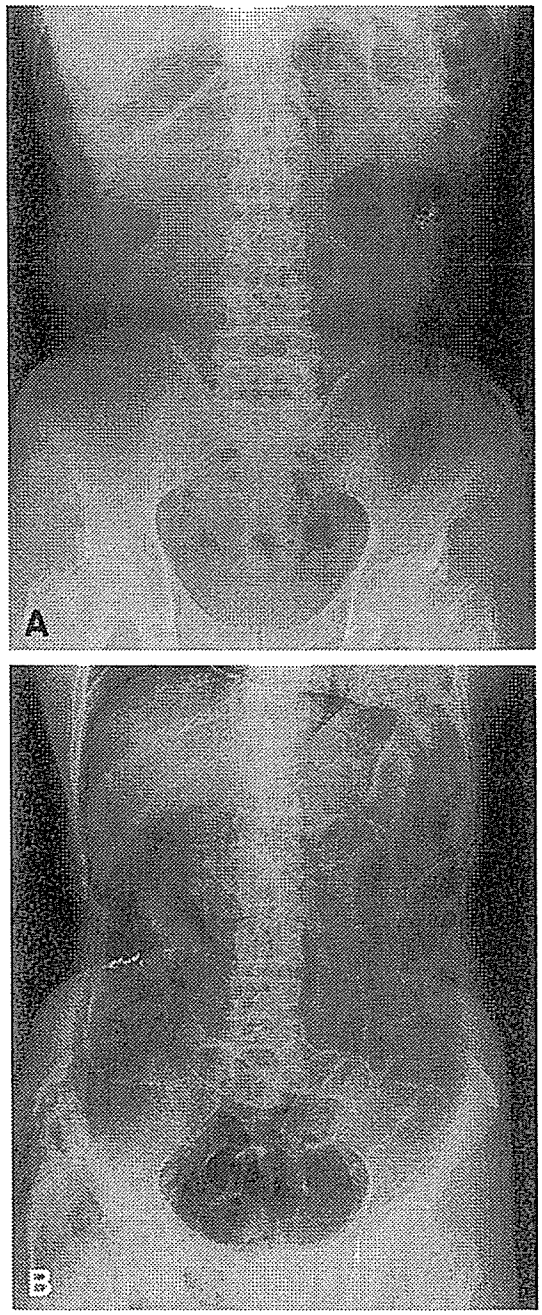

Figure 3. Abdominal radiographs after ESD. **A,** Less colonic gas in the CO_2 group than in the control group **(B)** is clearly shown. **B,** Control group.

which was considerably higher compared with that of conventional EMR techniques.[32-36]

Based on these results, we believe that colonic ESD is the preferable procedure for the en bloc resection of selected large flat neoplasia, such as nongranular type LSTs, but the operation time is much longer compared with the EPMR technique. It is important, therefore, to use CO_2 insufflation in colonic ESD procedures to reduce patient discomfort, and its safety and efficacy during long procedures was proven in this study.

CONCLUSIONS

This study strongly suggests that CO_2 insufflation is safe and effective during lengthy colonic endoscopic procedures (eg, ESD) with the patient under conscious sedation. It is possible, however, that a significant component of the reduced midazolam dosage in the CO_2 group might have been the technical improvement of the experienced endoscopists between the time of the control cases and the current series.

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DISCLOSURE

The authors have no commercial associations (eg, equity ownership or interest, consultancy, patent and licensing agreement, or institutional and corporate associations) that might be a conflict of interest.

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CANCER

Endoscopic indications for endoscopic mucosal resection of laterally spreading tumours in the colorectum

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Background: Laterally spreading tumours (LSTs) in the colorectum are usually removed by endoscopic mucosal resection (EMR) even when large in size. LSTs with deeper submucosal (sm) invasion, however, should not be treated by EMR because of the higher risk of lymph node metastasis.
Aims: To determine which endoscopic criteria, including high magnification pit pattern analysis, are associated with sm invasion in LSTs and clarify indications for EMR.
Methods: Eight endoscopic criteria from 511 colorectal LSTs (granular type (LST-G type); non-granular type (LST-NG type)) were evaluated retrospectively for association with sm invasion, and compared with histopathological findings.
Results: LST-NG type had a significantly higher frequency of sm invasion than LST-G type (14% *v* 7%; $p<0.01$). Presence of a large nodule in LST-G type was associated with higher sm invasion while pit pattern (invasive pattern), sclerous wall change, and larger tumour size were significantly associated with higher sm invasion in LST-NG type. In 19 LST-G type with sm invasion, sm penetration determined histopathologically occurred under the largest nodules (84%; 16/19) and depressed areas (16%; 3/19). Deepest sm penetration in 32 LST-NG type was either under depressed areas (72%; 23/32) or lymph follicular or multifocal sm invasion (28%; 1/32 and 8/32, respectively).
Conclusions: When considering the most suitable therapeutic strategy for LST-G type, we recommend endoscopic piecemeal resection with the area including the large nodule resected first. In contrast, LST-NG type should be removed en bloc because of the higher potential for malignancy and greater difficulty in diagnosing sm depth and extent of invasion compared with LST-G type.

The incidence of superficial colorectal tumours has been increasing recently. Among them, laterally spreading tumours (LSTs) typically extend laterally and circumferentially rather than vertically along the colonic wall[1] and the frequency of invasive carcinoma is lower than for polypoid lesions of similar size. LSTs are usually removed therefore by endoscopic mucosal resection (EMR) but larger such tumours may require piecemeal resection.[2–4] LSTs with deeper submucosal (sm) invasion, however, should not be treated by EMR due to the higher risk of lymph node metastasis. As a result, it is clinically important to accurately diagnose sm invasion before treatment.

We have previously reported that granular type LSTs (LST-G type) with large nodules or depressions tend to invade the sm.[2] Recently, pit pattern analysis using magnification colonoscopy has also been reported to be useful in the diagnosis of invasive depth in early colorectal cancers.[5–11] In addition, Kudo reported that there is another LST subtype with no granular pattern on the surface, referred to as non-granular type (LST-NG type),[1] and clinicopathological differences between LST-G type and LST-NG type have been reported.[3 5 12 13]

The aim of this study was to analyse endoscopic criteria including pit pattern analysis conducted using high magnification endoscopy from a large number of colorectal LSTs to determine the indications for EMR

MATERIALS AND METHODS

A total of 511 colorectal LSTs in 475 patients were resected endoscopically or surgically at the National Cancer Centre Hospital in Tokyo between January 1999 and December 2003.

In this study, LSTs were defined as lesions ≥10 mm in diameter with a low vertical axis extending laterally along the interior luminal wall. These lesions then were subdivided into two subtypes based on endoscopic macroscopic findings: LST-G type with even or uneven nodules on the surface and LST-NG type with a smooth surface (fig 1A, B). Patients excluded from the study were those who had advanced colorectal cancer, familial adenomatous polyposis, or inflammatory bowel syndrome.

When a lesion was detected by conventional endoscopic examination, surface mucous was washed away with lukewarm water containing pronase (Pronase MS; Kaken Pharmaceutical Co., Ltd, Tokyo, Japan) and then 0.4% indigo carmine dye was sprayed over the lesion in order to enhance its surface detail.

High magnification colonoscopy (CF-240ZI, PCF-240ZI, and CF-200Z; Olympus Optical Co., Ltd, Tokyo, Japan) was also used in this study. When high magnification observation with indigo carmine dye was not enough to determine the surface structure (pit pattern analysis), staining with 0.05% crystal violet was performed.[6]

Eight endoscopic criteria were investigated retrospectively for their possible association with sm invasion and each finding was then divided into two groups as follows:

(1) Tumour size: ≥20 mm or <20 mm; size was estimated for en bloc resected specimens by histopathological

Abbreviations: LST, laterally spreading tumour; LST-G type, LST granular type; LST-NG type, LST-non granular type; sm, submucosal; EMR, endoscopic mucosal resection; ESD, endoscopic submucosal dissection; EUS, endoscopic ultrasonography

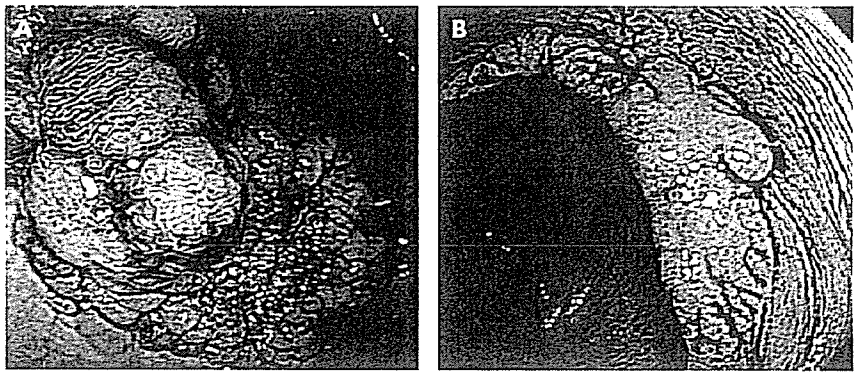

Figure 1 Laterally spreading tumour (LST) characteristics: (A) LST granular type. (B) LST non-granular type.

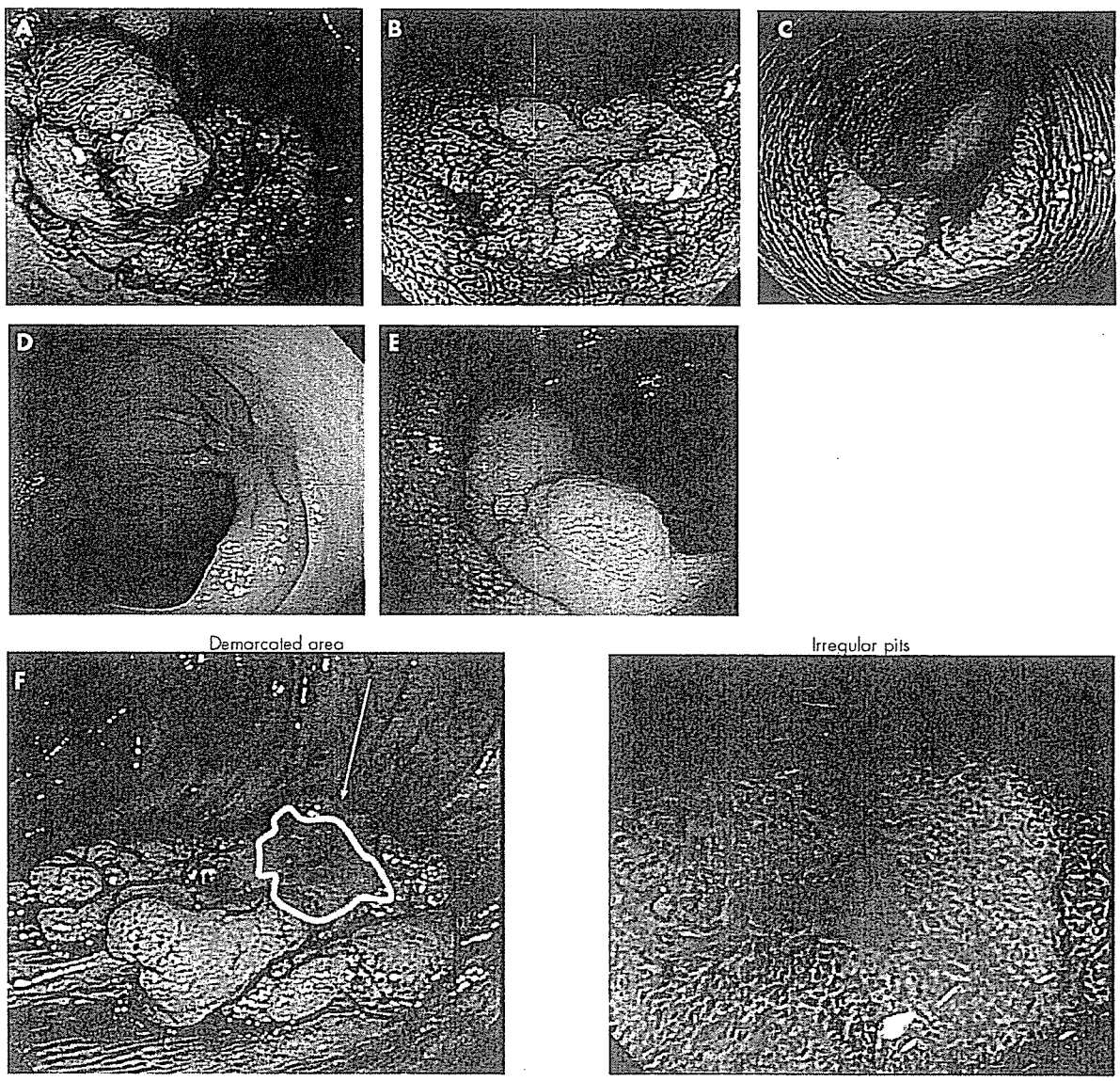

Figure 2 Endoscopic characteristics. (A) Large nodule (≥10 mm). (B) Demarcated depressed area (C) Sclerous wall change. (D) Fold convergency. (E) Chicken skin mucosa. (F) Pit pattern (invasive pattern).

Table 1 Clinicopathological characteristics of laterally spreading tumours of the granular (LST-G) and non-granular (LST-NG) types

	LST-G type	LST-NG type
No of LSTs	287	224
No of patients	275	195
Tumour size (mm) (mean (SD))	23.0 (12.6)	15.9 (7.0)
Histological diagnosis		
Adenoma or m-Ca	268 (93%)	192 (86%)
sm-Ca	19 (7%)	32 (14%)
Distribution		
Right colon	153 (53%)	136 (61%)
Left colon	42 (15%)	59 (26%)
Rectum	92 (32%)	29 (13%)

m-Ca, intramucosal adenocarcinoma.
sm-Ca, submucosal adenocarcinoma.

examination and for piecemeal resected specimens by reviewing endoscopic photographs.

(2) Surface redness: prominent or not prominent.

(3) Large nodule (≥10 mm): with or without such a nodule (fig 2A).

(4) Demarcated depressed area: with or without such a demarcation (fig 2B).

(5) Sclerous wall change: with or without such a change (fig 2C).

(6) Fold convergency: with or without such a fold convergency towards the tumour (fig 2D).

(7) Chicken skin mucosa: presence or absence of a chicken skin appearance around the tumour[14 15] (fig 2E).

(8) Pit pattern: "invasive pattern" or "non-invasive pattern"; "invasive pattern" is characterised by irregular and distorted epithelial crests observed in a demarcated area suggesting that sm invasion is more than 1000 μm, while "non-invasive pattern" does not have these two findings, suggesting intramucosal neoplasia or sm invasion less than 1000 μm[7-9 16] (fig 2F).

All endoscopic criteria were determined retrospectively by two highly experienced endoscopists (TU, YS), each of whom had previously performed over 1000 colonoscopies per year for more than five years. When there were any differences between their respective diagnoses, the two endoscopists discussed their findings and arrived at a single appropriate diagnosis.

In addition, the relationships between the various endoscopic criteria and the extent of sm invasion were analysed histopathologically in those LSTs with sm invasion.

Histopathology

Resected specimens were fixed in a 10% buffered formalin solution. Paraffin embedded samples were then sliced into 3 μm sections and stained with haematoxylin-eosin. Histopathological diagnosis was based on the Vienna classification.[17]

Statistical analysis

Endoscopic criteria were subjected to univariate and multivariate analysis. Data are presented as mean (SD). Data obtained were evaluated with the χ^2 and t tests using the SAS Statistical Package (SAS Institute, Tokyo, Japan). Significance level was set at 5% for each analysis. A p value <0.05 was considered significant.

RESULTS

Table 1 shows the clinicopathological characteristics of the LSTs. Although the mean size of LST-NG type (15.9 (7.0) mm) was smaller than that of LST-G type (23.0 (12.6) mm), the LST-NG type had a significantly higher frequency of sm invasion (14% (32/224) *v* 7% (19/287), respectively; $p<0.01$). LST-G type was more commonly diagnosed in the right colon and rectum (85% (245/287); 53% (153/287); and 32% (92/287), respectively). In contrast, LST-NG type was more frequently diagnosed in the right colon (61% (136/224); $p<0.001$).

In the LST-G type, the presence of a large nodule (≥10 mm), demarcated depressed area, invasive pattern, prominent redness, larger tumour size (≥20 mm), or sclerous

Table 2 Endoscopic criteria for submucosal invasion in 287 laterally spreading tumours, granular type

		Univariate analysis	Multivariate analysis	
	sm-Ca/n	p Value	Odds ratio	p Value
Large nodule				
≥10 mm	14/47	<0.001	71.01	<0.001
<10 mm	5/240			
Redness				
Present	6/17	<0.001	1.04	NS
Absent	13/270			
Depressed area				
Present	6/6	<0.001	28.66	NS
Absent	13/281			
Pit pattern				
Present	7/7	<0.001	>100	NS
Absent	12/280			
Tumour size				
≥20 mm	16/173	0.030	0.95	NS
<20 mm	3/114			
Sclerous wall change				
Present	2/2	0.004	2.48	NS
Absent	17/285			
Fold convergency				
Present	1/1	0.066	–	–
Absent	18/286			
Chicken skin mucosa				
Present	1/6	0.340	–	–
Absent	18/281			

sm-Ca, submucosal adenocarcinoma.

Table 3 Endoscopic criteria for submucosal invasion in 224 laterally spreading tumours, non-granular type

		Univariate analysis	Multivariate analysis	
	sm-Ca./n	p Value	Odds ratio	p Value
Sclerous wall change				
Present	13/14	<0.001	34.90	0.009
Absent	19/210			
Pit pattern				
Present	17/19	<0.001	16.46	0.020
Absent	15/205			
Tumour size				
≥20 mm	23/67	<0.001	8.98	0.001
<20 mm	9/157			
Depressed area				
Present	9/12	<0.001	9.70	NS
Absent	23/212			
Redness				
Present	8/8	<0.001	>100	NS
Absent	24/216			
Large nodule				
≥10 mm	2/2	0.020	>100	NS
<10 mm	30/222			
Fold convergency				
Present	3/7	0.062	–	–
Absent	29/217			
Chicken skin mucosa				
Present	7/28	0.087	–	–
Absent	25/196			

sm-Ca, submucosal adenocarcinoma.

wall change was significantly associated with an increased risk of sm invasion according to univariate analysis. Based on multivariate analysis, the only independent risk criterion for sm invasion was the existence of a large nodule (≥10 mm) (p<0.001; odds ratio 71.01) (table 2).

In comparison, the presence in LST-NG type of a sclerous wall change, invasive pattern, larger tumour size (≥20 mm), large nodule (≥10 mm), demarcated depressed area, prominent redness, or fold convergency was significantly associated with an increased risk of sm invasion according to univariate analysis. Based on multivariate analysis, independent risk criteria for sm invasion were the existence of a sclerous wall change, invasive pattern, or larger tumour size (≥20 mm) (p = 0.009, 0.020, and 0.001, respectively; odds ratios 34.90, 16.46, and 8.98, respectively) (table 3).

For LSTs with sm invasion, the presence of a large nodule (≥10 mm) in LST-G type had a higher accuracy and specificity than any other endoscopic criteria (table 4). A large nodule (≥10 mm) had a higher accuracy of sm invasion in LST-G type than sclerous wall change, invasive pattern, larger size, or any combination of these three risk criteria in LST-NG type (p<0.01; 87% *v* 77%).

In determining the extent of penetration histopathologically in the 19 LST-G type with sm invasion, the deepest penetration occurred under the largest nodule 84% of the time (16/19) and under the depressed area in the other three (16%; 3/19). In contrast, the deepest penetration in the 32 LST-NG type with sm invasion occurred under the depressed area (72%; 23/32) or consisted of lymph follicular or multifocal sm invasion (28%; 9/32; 1/32, and 8/32, respectively) (fig 3).

DISCUSSION

In this large study, we established that LST-NG type has a higher potential for malignancy than LST-G type. Based on this result and previous reports that LST-G type and LST-NG type differ in their genetic alterations,[12 13] we suggest that different therapeutic strategies are required for treating LSTs according to their specific macroscopic type (fig 4).

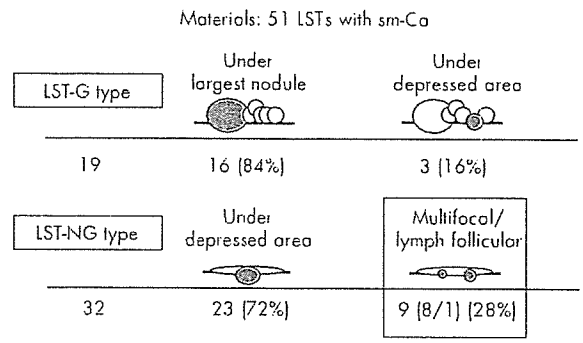

Figure 3 Histopathological analysis of submucosal penetration. LST-G type, laterally spreading tumour granular type; LST-NG type, laterally spreading tumour non-granular type; sm-Ca, submucosal adenocarcinoma.

Table 4 Accuracy rates of endoscopic criteria for submucosal invasion according to macroscopic type

	Accuracy	Sensitivity	Specificity	PPV
Large nodule (LST-G type)	87% (249/287)	74% (14/19)	88% (235/268)	30% (14/47)
Pit pattern or tumour size or sclerous wall change (LST-NG type)	77% (174/224)	88% (28/32)	76% (146/192)	38% (28/74)

LST-G type, laterally spreading tumour granular type; LST-NG type, laterally spreading tumour non-granular type; PPV, positive predictive value.

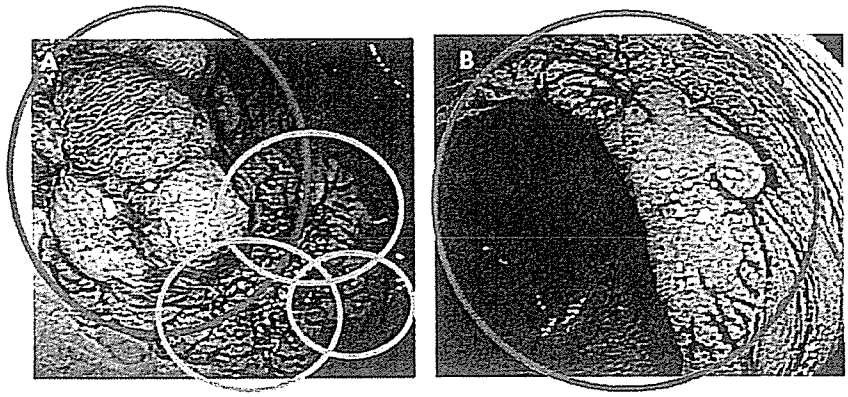

Figure 4 Endoscopic mucosal resection therapeutic strategies. (A) For colorectal laterally spreading tumour granular type. (B) For colorectal laterally spreading tumour non-granular type.

Pit pattern analysis using high magnification colonoscopy has been reported to be useful in the differential diagnosis of intramucosal and sm invasive cancers[7-11] and in differentiating non-neoplastic and neoplastic lesions.[16-20] Analysis of pit patterns also played an important part in this study.

Endoscopic ultrasonography (EUS) was not used to determine the depth of cancer invasion in this study because we previously reported that high magnification colonoscopy is superior to EUS for determination of invasion depth in early colorectal cancers.[16]

We investigated endoscopic criteria, including high magnification diagnoses of pit patterns, in order to predict sm invasion before treatment in a large number of two subtypes of LSTs, LST-G type and LST-G type, and found that there were differences in the endoscopic criteria for predicting sm invasion between these two LST subtypes. Multivariate analysis showed that an endoscopic criterion of a large nodule (≥10 mm) in LST-G type and the existence of a sclerous wall change, invasive pattern, or a larger tumour size (≥20 mm) in LST-NG type were independent risk criteria for sm invasion.

In this study, pit pattern high magnification diagnosis proved to be useful for predicting sm invasion in LSTs, particularly LST-NG type, although it was not as helpful with LST-G type. The existence of a large nodule in LST-G type was the most reliable risk criterion for determining sm invasion. Lack of pit pattern abnormalities in LST-G type patients may be due to the fact that some LST-G type with large nodules invade deeply into the sm layer without destructive surface changes compared with LST-NG type lesions.[21]

In considering therapeutic strategies, EMR should be the firstline treatment for most LSTs because of lower invasive carcinoma frequency compared with polypoid lesions of similar size. Lymph node metastasis is more frequently present in deeper sm invasive cancer.[22-24] As a result, we should avoid EMR for deeper sm invasive cancer because histological assessment is difficult, incomplete EMR is thought to cause accelerated growth of any residual cancer, and is also considered to be a positive risk factor for distant metastasis.[25 26] Recognising the importance of the reported endoscopic criteria for predicting sm invasion therefore is essential for determining the correct treatment choice in any given case.

The presence of deeper sm invasion in LST-G type was relatively low (6.6%; 19/287) with the vast majority (84%) of carcinoma invasions of the sm layer in LST-G type occurring under the largest nodule,[2] as revealed by histopathological examinations. Based on this, we recommend that the area including a large nodule in LST-G type should be resected first endoscopically followed by the remaining tumour (fig 4A).

In contrast, nearly 30% of LST-NG type revealed lymph follicular or multifocal sm invasion, both of which were difficult to diagnose by the indicated endoscopic criteria prior to treatment. Such tumours therefore should be removed en bloc to ensure an accurate histopathological diagnosis (fig 4B). As LST-NG type ≥20 mm are technically more difficult to remove en bloc by conventional EMR techniques[2 3 27] and LST-NG type have a higher malignancy potential than LST-G type, we believe that endoscopic submucosal dissection (ESD)[28-31] or laparoscopy assisted colectomy should be the techniques of choice for en bloc resection of LST-NG type ≥20 mm.

Although en bloc resection allows for accurate histological assessment, no residual tumour, and lower local recurrence, colorectal ESD is not widespread, even among Japanese colonoscopists because of its technical difficulty, longer procedure time, and increased risk of perforation compared with conventional EMR. Sano *et al* reported that the incidence of perforation in ESDs using an insulation tipped electrosurgical knife (IT knife) was considerably higher, occurring in 14.3% (2/14) of such cases.[30]

In order to reduce these negative factors, further refinements in devices such as the IT knife, improved submucosal injection solutions,[29] and better traction systems[29 31] will be necessary. While still in the developmental stages at the present time, it may be possible for colorectal ESD to become the standard procedure for all LSTs, regardless of their macroscopic type, as improvements facilitating easier, faster, and safer procedures are made in the future.

CONCLUSION

LST-G type and LST-NG type lesions differ both in terms of their frequency of sm invasion and their respective endoscopic criteria for predicting such sm invasion. In determining the appropriate therapeutic strategy therefore it is necessary to consider the most suitable treatment based on the specific macroscopic type of LST.

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Proteomic Signature Corresponding to the Response to Gefitinib (Iressa, ZD1839), an Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor in Lung Adenocarcinoma

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Abstract **Purpose:** We aimed to identify candidate proteins for tumor markers to predict the response to gefitinib treatment.

Experimental Design: We did two-dimensional difference gel electrophoresis to create the protein expression profile of lung adenocarcinoma tissues from patients who showed a different response to gefitinib treatment. We used a support vector machine algorithm to select the proteins that best distinguished 31 responders from 16 nonresponders. The prediction performance of the selected spots was validated by an external sample set, including six responders and eight nonresponders. The results were validated using specific antibodies.

Results: We selected nine proteins that distinguish responders from nonresponders. The predictive performance of the nine proteins was validated examining an additional six responders and eight nonresponders, resulting in positive and negative predictive values of 100% (six of six) and 87.5% (seven of eight), respectively. The differential expression of one of the nine proteins, heart-type fatty acid–binding protein, was successfully validated by ELISA. We also identified 12 proteins as a signature to distinguish tumors based on their *epidermal growth factor receptor* gene mutation status.

Conclusions: Study of these proteins may contribute to the development of personalized therapy for lung cancer patients.

Non-small cell lung carcinoma (NSCLC) accounts for ~85% of lung cancer cases (1). Biomarker(s) that predict the response to gefitinib (Iressa; AstraZeneca, Macclesfield, United Kingdom), an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, may help to improve the choice of therapeutic strategy in patients with NSCLC. Gefitinib improves NSCLC-related symptoms and quality of life in some patients with advanced NSCLC who do not respond to platinum-based chemotherapy. However, the response rate for gefitinib remains <20% in patients with NSCLC (2–4), and treatment with gefitinib is associated with serious adverse effects, such as severe acute interstitial pneumonia in 5.4% of the patients who received the treatment (5, 6). Thus, it is imperative to select appropriate patients for treatment with gefitinib and exclude patients in whom gefitinib is unlikely to exhibit any clinical benefit. Women, patients who have never smoked, patients with adenocarcinoma, and East Asians are major subgroups of responders (3, 4, 6–8). Recently, gain-of-function somatic mutation in the tyrosine kinase domain of the EGFR has been correlated with the response to gefitinib (9, 10). However, other studies have revealed that correction of the phenotype arising from EGFR mutation may not account for all of the clinical benefits of gefitinib (11, 12), and both preclinical and clinical studies have reported that the efficacy of gefitinib is independent of EGFR expression level (11, 13–15). Although molecular features of the *EGFR* gene, including mutation and high copy number, (16, 17) are associated with response to gefitinib, other molecular markers in the tumor, such as HER2 overexpression (18), Akt phosphorylation (19), and other EGFR downstream molecules (20), also correlate with response. These observations suggest a role for unknown, but important, factors in gefitinib sensitivity. Identification and elucidation of such factors will improve existing therapeutic protocols and contribute to further understanding of the mechanisms of gefitinib sensitivity.

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To identify the gene products correlated with the efficacy of gefitinib, genome-wide screening was done recently for NSCLC. A global mRNA expression study using DNA microarrays and biopsy samples identified 51 genes associated with the sensitivity to gefitinib and established a numerical scoring system to predict the response (21). This expression study also led to the establishment of ELISA assays for the identified gene products in serum. Preclinical studies involving mRNA profiling of NSCLC xenografts resulted in the identification of a set of genes that were differentially expressed between tumors that were sensitive and insensitive to gefitinib treatment (22, 23). These studies will lead to the identification of novel biomarkers to predict the response to gefitinib treatment. However, mRNA expression does not necessarily correlate with protein level, and posttranslational modifications, such as phosphorylation, cannot be predicted from the amount of RNA or from the DNA sequence (24). With this background, comprehensive expression studies at the protein level, an approach called proteomics, have been conducted in patients with lung cancer to develop biomarkers that predict clinical outcomes (25). However, no global protein expression study has yet been done on the mechanism of response to gefitinib.

To identify the proteomic signature for sensitivity to gefitinib and to use that signature as a tumor marker to predict the response to gefitinib, we analyzed global protein expression levels in lung adenocarcinoma tissues for whom we have detailed information on *EGFR* gene status. The surgical specimens were obtained at the time of surgery from patients who subsequently had recurrence and received gefitinib monotherapy. We then used two-dimensional difference gel electrophoresis (2D-DIGE) covering ~2,000 proteins to identify a set of proteins of which expression was associated with sensitivity to gefitinib and with EGFR mutation. The predictive performance of the protein set was validated with an independent data set and compared with that of EGFR mutation.

Materials and Methods

Patients and tissue samples. We examined tumor tissues from patients who relapsed after surgery and received gefitinib monotherapy. Two hundred seventy-nine patients who received gefitinib at the National Cancer Center Hospital from July 2002 to December 2004 were evaluated for inclusion in this study. Ninety-two patients relapsed after surgical resection of primary NSCLC and started to receive monotherapy with gefitinib 250 mg/d for 14 days (n = 92). We used tumor tissues obtained at the time of surgery and stored in vapor nitrogen. Fifteen patients were excluded from our study for the following reasons: frozen tissues were not available (n = 10) and tumor histology showed squamous cell carcinoma (n = 4) or pleomorphic carcinoma (n = 1). The histologic features of the tissues were reviewed by two board-certified pathologists (Y.M and K.T.) and diagnosis was based on the latest WHO classification of lung adenocarcinoma (8, 26–28). The tumor responses were classified into complete response (CR), partial response (PR), and progressive disease (PD) using standard bidimensional measurements (29). In this study, patients without a marked reduction of tumor size were subdivided into minor response (MR) and stable disease (SD) groups. MR was defined as a 25% decrease in the sum of the products of perpendicular diameters of all measurable lesions at any point during gefitinib treatment. SD was defined as a <25% decrease in tumor size after treatment. The clinical information is summarized in Table 1, and further information, including EGFR mutation status, is summarized in Supplementary Table S1. Consent was obtained from all patients and the protocol was approved by the institutional review board of the National Cancer Center.

To identify the proteins associated with response to gefitinib, we compared the protein expression profiles of responders (CR and PR) and nonresponders (PD). Of 77 samples available, the effects of gefitinib treatment were not examined for six cases because the treatment was not completed. These six samples were excluded from this study. We constructed two sample sets in the following way (Table 2): a training sample set comprising 31 responders (2 CRs + 29 PRs) and 16 nonresponders (16 PDs) and a test set comprising six responders (6 PRs) and 8 nonresponders (8 PDs) from whom samples were obtained between June and December 2004 (Table 2). As no significant differences were observed between CRs and PRs (Supplementary Fig. S1A), we grouped CRs and PRs together in the responder group.

Protein extraction and protein expression profiling. The frozen tumor tissues were crushed to frozen powder with a Multi-Beads Shocker (Yasui-kikai, Osaka, Japan) under cooling with liquid nitrogen. The frozen powder was then treated with urea lysis buffer (7 mol/L urea, 2 mol/L thiourea, 3% CHAPS, 1% Triton X-100) for 30 min on ice. After centrifugation at 15,000 rpm for 30 min, the supernatant was recovered as cellular protein for the protein expression study.

Protein samples were labeled with CyDye DIGE Fluor saturation dye (GE Healthcare Amersham Biosciences, Uppsala, Sweden) according to

Table 1. Patient characteristics

	No. patients	%
Gender		
Female	33	43
Male	44	57
Age (y)		
Median (range)	62.2 (32-80)	—
Histologic type		
Adenocarcinoma		100
Papillary/acinar/ bronchioloalveolar/solid	30/16/9/6	49/26/15/10
Smoking history*		
Never smokers	37	48
Former smokers	12	16
Current smokers	28	36
ECOG performance status†		
0/1/2/3	24/39/9/5	31/51/12/6
Prior chemotherapy		
Yes	30	39
No	47	61
Response to gefinitib		
CR/PR/MR/SD/PD/NE	2/35/2/8/24/6	3/45/3/10/31/8
EGFR gene status		
Mutation L858R	18	23.4
DEL‡	18	23.4
G719§	2	2.6
Wild-type	35	45.4
Unknown	4	5.2

Abbbreviation: NE, not evaluated.
*Never-smokers: those who had never had a smoking habit; former smokers: those who had stopped smoking at least 1 yr before diagnosis; and current smokers: active smokers at diagnosis of NSCLC or those who had stopped smoking less than 1 yr before diagnosis.
†ECOG performance status was monitored according to the previous report (44).
‡Deletional mutations in exon 19.
§G719S and G719C.

Table 2. Training and test sets to develop the classifier for the response to gefitinib

	Training set			Test set		
	Responders, n = 31 (%)	Nonresponders, n = 16 (%)	P	Responders, n = 6 (%)	Nonresponders, n = 8 (%)	P
Age						
Mean ± SD	64.0 ± 8.9	60.5 ± 12.0	0.330	57.5 ± 12.8	62.8 ± 6.1	0.386
Gender						
Male	17 (55)	9 (56)	0.927	3 (50)	5 (62.5)	0.640
Female	14 (45)	7 (44)		3 (50)	3 (37.5)	
Smoking history						
Never smokers	17 (55)	9 (56)	0.286	4 (67)	4 (50)	0.054
Former smokers	7 (22.5)	1 (6)		2 (33)	0 (0)	
Current smokers	7 (22.5)	6 (38)		0 (0)	4 (50)	
EGFR gene status						
Mutation	27 (87)	1 (6)	<0.001	4 (66)	0 (0)	0.006
Wild type	3 (10)	13 (81)		1 (17)	8 (100)	
Unknown	1 (3)	2 (13)		1 (17)	0 (0)	
Prior chemotherapy						
(+)	12 (39)	5 (31)	0.614	6 (100)	0 (22)	<0.001
(−)	19 (61)	11 (69)		0 (0)	8 (100)	
Performance status						
0	11 (35.5)	6 (37.5)	0.945	2 (33)	1 (12.5)	0.347
1	11 (35.5)	10 (62.5)		4 (67)	7 (87.5)	
2	6 (19)	0 (0)		0 (0)	0 (0)	
3	3 (10)	0 (0)		0 (0)	0 (0)	

our previous report (30). We prepared an internal control consisting of a mixture of small portions of all protein samples obtained before May 2004 (31). The internal control sample and the individual experimental samples were labeled with Cy3 and Cy5 CyDye DIGE Fluor saturation dyes, respectively. Five micrograms of Cy3- or Cy5-labeled protein were mixed and coseparated by two-dimensional PAGE. The first-dimension separation was achieved on an Immobiline pH gradient gel (isoelectric point range, 4-7; 24 cm length) with a Multiphor II (GE Healthcare Amersham Biosciences). The second-dimension separation was done with an EttanDalt II (GE Healthcare Amersham Biosciences) with a 9% to 15% gradient polyacrylamide gel. After electrophoresis, the gels were scanned at appropriate wavelengths for Cy3 and Cy5 (Supplementary Fig. S2A). The ratio between Cy5 and Cy3 intensity was calculated for all protein spots in identical gels by the use of DeCyder software (GE Healthcare Amersham Biosciences; ref. 31). The standardized spot intensities were then logarithmically transformed and subjected to a data-mining package (Impressionist; GeneData, Basel, Switzerland). We ran triplicate gels for each sample and calculated the averaged standardized spot intensity.

To assess the reproducibility of the proteomic data with the internal control in our analyses, we generated triplicate protein profiles from identical samples (case 9; Supplementary Table S1) and compared the standardized intensity of the paired spots (Supplementary Fig. S2B). Scattergrams with 1,980, 1,646, and 1,873 spots showed that the intensities of 1,916 (93.7%), 1,599 (94.7%), and 1,770 (94.5%) spots, respectively, were scattered within a 2-fold difference, and the correlation values were also high (r values > 0.93; Supplementary Fig. S2B).

Data analysis. A bioinformatic approach based on a support vector machine (SVM) algorithm and a leave-one-out cross-validation was used to identify proteins of which expression was associated with tumor characteristics, including therapeutic response to gefitinib and the presence of EGFR mutation (32).

Protein identification. Proteins corresponding to the protein spots of interest were identified by mass spectrometry (30). The proteins were recovered in a gel plug by using an automated spot collector (SpotPicker; GE Healthcare Amersham Biosciences) and digested with sequence grade trypsin (Promega, Madison, WI; ref. 30). Trypsin digests were applied to liquid chromatography coupled with tandem mass spectrometry (LTQ, Thermo, Waltham, MA). A database search against Swiss-Prot was done with Mascot software. Patients with a Mascot score of 35 or more were used for protein identification. When multiple proteins were identified in a single spot, the proteins with the highest number of peptides were considered as those corresponding to the spot.

Mutations in the EGFR gene. EGFR mutations in the samples obtained between July 2002 and May 2004 were examined as described in our previous report (8). Analysis of samples obtained between June 2004 and December 2004 was done by high-resolution melting analysis with a LightCycler HR-1 system (Idaho Technology Inc., Salt Lake City, UT).

ELISA. The expression level of heart-type fatty acid–binding protein (H-FABP) in protein samples from 55 lung adenocarcinoma patients (2 CRs, 28 PRs, 6 SDs, 1 MR, and 18 PDs) was measured in a clinical laboratory (SRL, Tokyo, Japan) with a commercially available ELISA kit (MARKIT-M H-FABP, Dainippon Pharmaceutical, Tokyo, Japan) according to the manufacturer's instructions (Supplementary Table S1). All these 55 samples were included in a 2D-DIGE analysis set in this study.

Results

Proteomic signature for the response to gefitinib. We first selected 1,685 protein spots that appeared in at least 80% of the images of Cy3-labeled internal control. We further selected 87 protein spots that showed different intensities between responder and nonresponder groups ($P < 0.05$, Wilcoxon test). Although potentially resulting in a loss of information, this trimming process decreased the possibility that the classifier would be significantly influenced by irrelevant expression data. We selected protein sets for which expression was associated with response to gefitinib by using a SVM algorithm. Accuracy, plotted as a function of spot number, was constant until the number of spots decreased to less than nine, showing that accurate classification did not require all protein spots (Fig. 1A). The location on the two-dimensional map is shown for the selected nine spots (Fig. 1B; Supplementary Fig. S3).

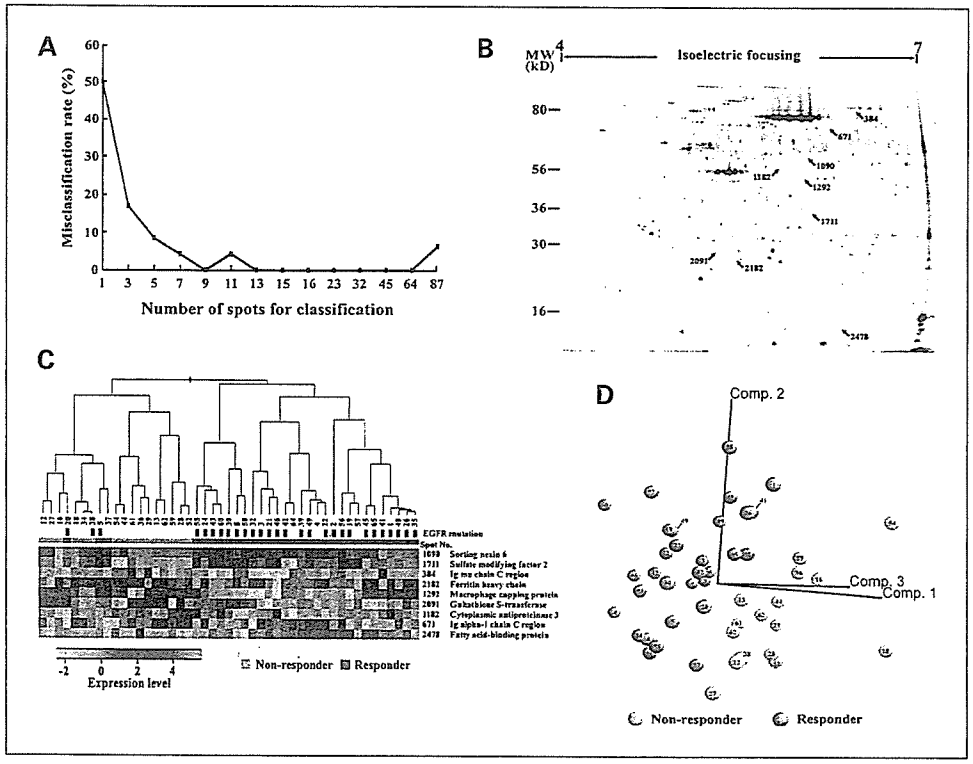

Fig. 1. Data-mining procedure to develop the prediction model for the response to gefitinib. *A*, a spot ranking method selected a few protein spots by which the cumulative error rate of a leave-one-out cross-validation became minimal. The spot ranking method indicated that the error rate was minimal when the prediction model was constructed by a particular nine protein spots. *B*, localization of the selected nine protein spots on the two-dimensional map. An enlarged two-dimensional image is shown in Supplementary Fig. S2. *C*, hierarchical clustering analysis of the samples in the learning set using the selected nine protein spots. Black bars, the presence of EGFR mutations within exons 18 to 21. *D*, principal component analysis of the samples in the learning set using the selected nine protein spots. Comp.1, 2, and 3, the first component 1, 2, and 3, respectively.

Mass spectrometry revealed that these nine spots corresponded to nine gene products (Table 3). Overall similarity of the selected spots is shown in Supplementary Fig. S1B and C. As the responder group in the training set consisted mainly of PRs, the obtained proteomic signature would presumably be more reflective of PR than CR.

The classification performance of the selected nine protein spots was validated by unsupervised classification. Hierarchical clustering showed that all tumor samples in the training set, except for cases 5, 20, and 37, were grouped according to their sensitivity to gefitinib based on the expression pattern of the nine proteins (Fig. 1C). In principal component analysis, all 47 samples seemed to be separated into two groups, although the border between these groups was not clear (Fig. 1D). Although hierarchical clustering and principal component analysis are crude methods of validation of classification, the results obtained using them were consistent.

To validate the predictive performance of the nine protein spots, we investigated a newly enrolled test sample set that was completely independent of the learning set. Based on the expression level of the nine protein spots, the distance of each sample from the hyperplane created by the SVM algorithm, defined as the SVM value, was calculated. The samples with a positive SVM value were grouped as responders and the samples with a negative SVM value were grouped as nonresponders. As a consequence, all training set samples were correctly classified in accordance with their clinical response to gefitinib (Fig. 2). All responders (six PRs) and seven of eight nonresponders (eight PDs) in the test set were also correctly classified. The expression pattern of the nine protein spots in the nonresponder patient (case 75) was more similar to that of the responder group, for unknown reasons. We also validated the results using the samples from patients who showed MR and SD. We found that the two patients showing MR were categorized as responders and that among the eight patients showing three SDs were classified into the responder group and five SDs into the nonresponder group. We did a leave-one-out cross validation for all 47 samples in the training set and the test set using nine protein spots with 1,000 times random permutation. All but two cases, cases 37 and 75, were correctly classified according to their status of response to the treatment. The overall misclassification error rate was 3.3%. Consequently, the model predicted the response to gefitinib in 13 of the 14 (92.8%) newly enrolled samples from the responders and nonresponders and may be useful for disease monitoring.

Proteomic signature for EGFR gene mutation. We studied the spots on the prediction for EGFR mutation. We set a training sample set, including 58 samples (34 mutation-positive samples and 24 mutation-negative samples; Supplementary Table S2). We found that the 12 protein spots showed the high correlation with the EGFR mutation (Supplementary Data; Supplementary Figs. S4-6). The classification and prediction performance of the selected 12 protein spots was successfully validated using the external validation sample set, including four mutation-positive samples and 11 mutation-negative samples (Supplementary Fig. S7). Only one protein, sulfate modifying factor 2, was shared between the signatures for the response and for the mutation (Table 3; Supplementary Table S3).

Expression of H-FABP measured by ELISA. We validated the differential expression of the identified proteins by the use of a widely available clinical assay. The expression level of H-FABP in the same tumor samples as those used in 2D-DIGE was measured with a commercially available ELISA kit intended for serum assays (Fig. 3). H-FABP expression measured by ELISA was highly correlated with that measured by 2D-DIGE (Pearson correlation, 0.76295; $P < 0.0001$). The ELISA study also showed